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13. ABSTRACT *(Maximum 200 words)*

Overexpression of HER family members and c-Src occurs in a majority of human breast cancers, suggesting the two tyrosine kinases may cooperate during neoplastic transformation. Synergism between c-Src and HER1 in tumorigenesis has been demonstrated in a murine fibroblast model and supported by studies in human breast cancer cells. To investigate whether c-Src/HER2 interactions may also play a role in breast tumor progression, we characterized a panel of thirteen human breast carcinoma cell lines and thirteen tumor samples for expression levels of HER family members and c-Src and for the *in vivo* presence of c-Src/HER2 heterocomplexes. A subset of the lines were examined for their growth rates in reduced serum, their ability to form colonies in soft agar in response to heregulin (HRG) and the requirement of c-Src kinase activity in these processes. By immunoblotting, in ~90% of the cell lines and tumor tissues tested, either HER1 or HER2 was overexpressed, while c-Src was overexpressed in >70% of the samples. HRG potentiated low-serum growth in five of six cell lines tested. Colony formation in soft agar was potentiated by HRG in three of the six, but only in those cell lines which exhibited a c-Src/HER2 heterocomplex, suggesting that physical association between c-Src and HER2 promoted HRG-potentiated, anchorage-independent growth. HRG effects were either partially or completely ablated by PP1, a Src family kinase inhibitor, which induced apoptosis after long-term treatment. Partial ablation could be accounted for by an anti-apoptotic effect of HRG. Taken together, these data indicate HRG can function co-operatively with and independently of c-Src to promote survival and growth of human breast tumor cells.

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Allison Babcock 8/8/98

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INTRODUCTION

HER2/*neu* is a member of the human epidermal growth factor (EGF) receptor (HER) family that has potent oncogenic activity in both human and rodent cells when overexpressed or activated by a single point mutation in the transmembrane domain or by delection of the first 621 amino acids from the N-terminus (human (Pierce, *et al.* 1991) and rat (DiFiore *et al.*, 1987)). Overexpression of HER2 is observed in a number of human cancers, including breast (Slamon *et al.*, 1987, Slamon *et al*, 1989), ovarian (Berchuck, *et al.*, 1990), gastric (Kameda *et al.*, 1990), lung (Kern *et al.*, 1990) and prostate (Ware *et al.*, 1991). In breast cancers, overexpression and/or amplification of HER2, which occurs in ~ 30 percent of the cases, has been correlated with poor prognosis (Slamon *et al.*, 1987; Slamon *et al.*, 1989). Overexpression of HER2 can function as a causal factor for breast cancer development, as has been demonstrated by targeted overexpression of HER2/*neu* and development of mammary tumors in mice (Muthuswamy *et al*, 1994).

The HER family of tyrosine kinases consists of four members, the EGF receptor (EGFR or HER1), HER2/*neu,* HER3 and HER4. HER1 has been shown to bind and become activated by different members of the EGF family of ligands, including EGF, heparin-binding EGF (HB-EGF), TGF-α, amphiregulin, epiregulin and betacellulin (Riese *et al.*, 1996; Riese *et al.*, 1998; Barnard *et al.*, 1994; Barnard, 1993; Komurasaki et al., 1997). HER4 also binds betacellulin (Barnard *et al.,* 1994), as well as epiregulin (Riese *et al.*, 1998), and HER3 and HER4 each act as receptors for all members of the heregulin family (also known as neuregulin and neu differentiation factor)(Carraway and Cantley, 1994; Hynes and Stern, 1994). In addition, EGF can bind HER4 (Wang et al., 1998) in cells engineered to overexpress HER4 and HER2, while HER2/HER3 heterodimers can act as receptors for both EGF and betacellulin (Pinkas-Kramarski *et al.,* 1998; Alimandi *et al.,* 1997).

While it shares a high percentage of homology with the HER family of receptor tyrosine kinases (80% sequence homology between HER1 and HER2, Coussens *et al.*, 1985), no ligand has been identified for HER2. However, HER2 can be activated through heterodimerization with HER3 and HER4, which bind the peptide ligand heregulin (HRG or NDF) (Holmes *et al.*, 1992; Wen *et al.*, 1991). Upon binding one of these receptors, HRG induces either homodimerization of HER3 or HER4 or heterodimerization of HER3/HER2 or HER4/HER2 (Carraway and Cantley, 1994; Carraway, III, *et al.*, 1995; Sliwkowski *et al.*, 1994; Plowman *et al.*, 1993a, 1993b). Dimerization is followed by trans-phosphorylation between paired receptor monomers and stimulation of PI_3 kinase and other signaling molecules (Carraway *et al.*, 1995; Sepp-Lorenzino *et al.*, 1996; Segatto *et al.*, 1993; Dankort *et al.*, 1997). Stimulation of cells with EGF is also believed to cause heterodimerization between HER1 and HER2, in addition to homodimerization of HER1 (Ullrich and Schlessinger, 1990). Studies in the 32D macrophage and chinese hamster ovary (CHO) cell lines, which express no endogenous HER family receptors, have indicated that formation of the HER2/HER3 heterodimer is preferred among all the receptor combinations and is the most tumorigenic (Tzahar *et al.*, 1996; Tzahar *et al.*, 1997; Pinkas-Kramarski *et al.*, 1996; Wallasch *et al.*, 1995; Karunagaran *et al.*, 1996). In this heterodimer, HER3 (which exhibits reduced intrinsic catalytic activity (Guy *et al.*, 1994)) appears to provide ligand binding activity (Carraway and Cantley, 1994) and the ability to associate with phosphatidylinositol-3 kinase (PI_3 kinase) (Gamett *et al.*, 1995; Soltoff *et al.*,

1994), while HER2 contributes kinase activity and the ability to phosphorylate downstream substrates as well as the ability to associate with PI_3 kinase.

Recently, several laboratories have reported that functional interactions between members of the HER receptor tyrosine kinase family and c-Src, a non-receptor tyrosine kinase, may play a role in breast tumor formation and progression. Elevated levels of c-Src protein or activity (Ottenholf-Kalff *et al.*, 1992), as well as stable association between c-Src and HER1 or HER2, have been observed in a high percentage of human cell lines and mouse mammary tumors (Muthuswamy *et al.*, 1994, Luttrell *et al.*, 1994; Muthuswamy and Muller, 1995; Maa *et al.*, 1995). In a murine fibroblast model, overexpression of c-Src was found to potentiate tumorigenesis induced by overexpression of HER1. This potentiation correlated with the EGF-inducible formation of a stable complex between c-Src and HER1, the appearance of two novel tyrosine phosphorylation sites on the receptor, and enhanced phosphorylation of receptor substrates, suggesting that when both c-Src and HER1 are overexpressed (as in human breast cancer cells), c-Src functionally synergizes with the receptor by phosphorylating and hyperactivating it (Maa *et al.*, 1995; Biscardi, *et al.*, 1998, submitted). The relevance of this model for human neoplasias is supported by an analysis of panels of human breast cancer cell lines and tumor tissues, in which overexpression of both c-Src and HER1 was found to correlate with the ability to detect a stable heterocomplex between c-Src and HER1, the appearance of the same two novel phosphorylations on the receptor, elevated tyrosine phosphorylation of downstream targets of HER1, and enhanced tumor formation in nude mice (Biscardi *et al.*, 1998). Together, these results imply that structural and functional interactions between c-Src and HER1 play critical roles in breast cancer tumor progression.

To determine if c-Src could functionally interact with HER2 as it does with HER1, one approach was to examine a panel of human breast tumor cell lines and tumor tissues for levels of HER family members and c-Src and for evidence of physical and functional interactions between HER2 and c-Src. HRG was used as a ligand to activate HER2 through HER3 or HER4 and to test tumor cells for their HRG-potentiated growth rates and tumorigenic properties in the presence or absence of the Src family kinase inhibitor, PP1 (Hanke *et al.*, 1996). We found that growth in low serum was potentiated to varying extents by HRG in five of six cell lines tested and that this growth enhancement was partially or completely reversed by PP1. Colony formation in soft agar was also potentiated by HRG, but only in those cell lines in which stable c-Src/HER2 complexes could be detected. As with growth in reduced serum, soft agar colony formation was either partially or completely inhibited by PP1. Interestingly, extended PP1 treatment was found to induce apoptosis, and in some cell lines, HRG partially rescued this effect in a wortmannin-sensitive fashion. Taken together, these data provide evidence for both co-operative and independent pathways involving c-Src and HER2 in HRG-mediated growth promotion.

A second approach to test possible functional interactions between HER2 and c-Src was to overexpress HER2 and c-Src, either together or alone, in C3H10T1/2 murine fibroblasts as a model system. A subset of these cell lines have been assayed for physical association of HER2 and c-Src, and remain to be assayed for changes in tumorigenic properties, changes in phosphorylation of the receptor, and changes in the receptor phosphorylation of downstream target molecules. Since interactions between HER2 and c-Src will be assayed in a simplified model system, it will be possible to correlate significant tumorigenic changes with the overexpression of a distinct protein in an established cellular background. Comparisons of the

tumorigenicity of wildtype and kinase-deficient HER2 in the model system may also allow for a direct test of cause and effect of the overexpression of HER2. Performed both in model systems and in breast cancer cell lines, these investigations will identify cellular molecules active in breast cancer formation and progression, as well as potential molecules to target in breast cancer therapeutic strategies.

MATERIALS AND METHODS

Cell lines. Tumor cell lines MDA-MB-468, MDA-MB-231, MCF7 and ZR75-1 were obtained from N. Rosen (Sloan-Kettering, New York), while MDA-MB-175, UACC-893, UACC-812, SK-BR-3, MDA-MB-361, MDA-MB-453, BT-474, BT-549, BT-20 and HS578Bst were obtained from American Type Culture Collection (Rockville, MD). Tumor cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Bethesda, MD) in 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA). Where indicated, cells were stimulated with recombinant human HRG-α, EGF-like domain (HRG-α) (200ng/ml) (Sigma Chemical, St. Louis, MO) or HRG-β1 (extracellular domain) (NeoMarkers, CA). HRG-α and HRG-β1 gave nearly identical results and were used interchangeably. The derivation and characterization of neomycin-resistant (Neo control) and c-Src overexpressor (5Hd47) C3H10T1/2 mouse fibroblasts have been described previously (Luttrell *et al.*, 1988; Wilson *et al.*, 1989; Maa *et al.*, 1995). C3H10T1/2 cell lines were maintained in DMEM with 10% fetal calf serum and 400μg/ml G418.

Overexpression of HER2 in C3H10T1/2 mouse fibroblasts. HER2 cDNA in a retroviral vector construct (LTR-1) (gift of P. DiFiore) (DiFiore *et al.*, 1987) were co-transfected along with pBABE puromycin-resistance vector (Morgenstern and Land, 1990) via Lipofectin reagent (Life Technologies, Gaithersburg, MD, USA), into Neo (control) and 5Hd47 (c-Src overexpressors). Transfected colonies were selected with G418 (400 μg/ml) and puromycin (concentration). Mutants of HER2 transfected include activated, oncogenic HER2 (V659E), kinase-deficient HER2 (L753R), a potential auto-phosphorylation site mutant (Y877F), and a chimeric receptor containing the extracellular domain of HER1 (residues -24->+621 from the original sequence, Ullrich, *et al.*, 1984) with transmembrane and intracellular domains of the HER2 receptor (residues +654->+1255 from Coussens, *et al.*, 1985). Overexpression of HER2 and/or c-Src proteins was assayed by Western blotting, and fold increases over parental cell lines determined by scanning by a Molecular Dynamics densitometer (San Francisco, CA, USA).

Growth assays. Cell lines were plated in triplicate in DMEM plus 0.5% fetal calf serum at a density of either $2x10^4$ cells/well (MDA-MB-468, MCF7) or $5x10^4$ cells/well (UACC-812, SK-BR-3, MDA-MB-361, MDA-MB-453) in 24-well tissue culture clusters (Corning, NY). Cells were allowed to adhere overnight and then incubated at 37°C in a humidified, 5% CO_2 atmosphere for five days with one of the following preparations in DMEM: 0.5% serum alone; 0.5% serum + 40ng/ml HRG-α or -β1; 0.5% serum + HRG + 10μM PP1, a c-Src family kinase inhibitor (CalBiochem, San Diego, CA); or 0.5% serum + PP1. Media with additives was replenished every 3 days. Cell number was assayed by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) treatment as per Kaspers *et al.*, 1991.

Soft agar assays were performed as described by Maa *et al.*, 1995. Cells were plated at a density of $1x10^4$ cells in triplicate in 60mm dishes, in the presence or absence of HRG-α

(40ng/ml), and in the presence or absence of PP1 (10μM). Plates were incubated for two weeks, with replenishment of appropriate media every 4 days, and stained overnight at 37°C, 5% CO_2, with 1μg/ml iodonitrotetrazolium salt (Sigma Chemical, St. Louis, MO). Colonies were counted using EagleSight analysis software (Stratagene, La Jolla, CA).

Antibodies. C-Src antibodies that were used in this study include mouse monoclonal antibodies (mAb) 2-17, directed against amino acids 2-17 (Quality Biotech, Camden, NJ); mAbs GD11 and EB8, both directed against residues 92-128 in the SH3 domain (Parsons *et al.*, 1984; Parsons *et al.*, 1986); and mAb 327, which recognizes the SH3 domain (gift from J. Brugge). HER2-specific antibody (rabbit polyclonal), directed toward residues 1169-1186, and HER3-specific antibody, directed toward residues 1307-1323, were obtained from Santa Cruz Biotechnologies, Santa Cruz, CA, USA. Antibodies specific for HER1, mAbs 3A and 4A, were provided by D. McCarley and R. Schatzman of Syntex Research, Palo Alto, CA. Their epitopes map to amino acid residues 889-944 and 1052 and 1134, respectively (Maa, 1995). Anti-phosphotyrosine (p-Tyr) polyclonal antibody (RC20) was purchased from Transduction Laboratories (Lexington, KY). Negative control antibodies included purified normal rabbit or mouse immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA, USA). SHC polyclonal antibody was purchased from UBI (Lake Placid, NY, USA). Anti-phospho-MAPK antibody was purchased from Promega (Madison, WI, USA), and anti-pan MAPK antibody (B3B9) was the gift of M. Weber (Reuter *et al.*, 1995). Negative control antibodies include purified normal rabbit or mouse immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA, USA).

Immunoprecipitation and Western immunoblotting. Cells were lysed in RIPA detergent buffer (10mM Tris-HCl pH 7.2, 1% Triton-X, 0.5% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM sodium orthovanadate, 50μg/ml leupeptin and 0.5% aprotinin). Tumor samples were minced with a scalpel and ground in a Dounce homogenizer in RIPA buffer, all performed at 4-10°C. Lysates were cleared by centrifugation in a microcentrifuge, and protein concentration was determined by BioRad protein reagent. For immunoprecipitation, 5 μg antibody was incubated with 500 μg cell lysate protein with continuous rocking at 4°C for 2 hr and then incubated for an additional hour at 4°C with protein A-sepharose beads (Sigma Chemical). After incubation, samples were centrifuged, and the sepharose beads were washed three times with cold RIPA buffer. Pellets were resuspended in 2X sample buffer (125mM Tris-HCl, 4% SDS, 10% glycerol, 0.02% bromophenol blue, 4% mercaptoethanol) and boiled for 5 min. Eluted proteins were separated by electrophoresis through a 7% SDS-polyacrylamide gel and immunoblotted according to previously published protocols (Luttrell *et al.*, 1988; Wilson *et al.*, 1989; Maa *et al.*, 1995). Binding of primary antibodies was visualized by ^{125}I-protein A, used at 1 μCi/ml (New England Nuclear, USA), or by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, England).

Apoptosis Assays. Quantitation of apoptosis on the single-cell level was performed by staining of cell nuclei with di-(amidinophenyl)-indole (DAPI)(Villaneuva *et al.*, 1984). MDA-MB-361, UACC-812 and MCF7 cells were plated onto glass coverslips in 6-well dishes (Corning, NY), and incubated with one of the following treatments: 0.5% serum alone; 0.5% serum + 40ng/ml HRG-α; 0.5% serum + HRG + 10μM PP1; 0.5% serum + PP1; 0.5% serum + 4nM wortmannin; 0.5% serum + HRG-α + wortmannin; 0.5% serum + HRG + PP1 + wortmannin; 0.5% serum + PP1 + wortmannin. UV-B treatment (1 minute, followed by 48 hours recovery in serum-free media) was used as a positive control for apoptosis. After three days treatment, cells were

serum-starved overnight, fixed in 4% paraformaldehyde, quenched in 0.25mg/ml H_3BO_4, 20mg/ml glycine and permeabilized in 0.5% Triton-X on ice. DAPI (1.0 μg/ml) (Sigma Chemical) was added for specific DNA staining.

RESULTS

Development of stable HER2 overexpressing cells. The development and characterization of wildtype and mutant HER2-overexpressing cell lines in the C3H10T1/2 mouse fibroblast system has progressed, both in the presence and absence of the overexpression of c-Src. Five of ten model cell lines have been obtained (Table I). Using electroporation, we have isolated many clones of wildtype HER2, but it has been difficult to demonstrate expression of the HER2 protein by Western blotting. By metabolically labeling these clones with ^{35}S-methionine, we can detect newly synthesized full-length protein by immunoprecipitating with human-specific HER2 antibodies (Oncogene Science). However, we hypothesize that the receptors are dimerized and activated, and are being internalized and degraded at a more rapid rate than endogenous receptors, and therefore not detectable by Western blotting. Future experiments will include chloroquine treatment to prevent lysosomal degradation of the receptors.

We have examined the three cell lines which overexpress c-Src and kinase-deficient HER2 for heterocomplex formation, and in each of these cell lines, we have demonstrated that HER2 co-immunoprecipitates with c-Src using the monoclonal antibody 2-17 to c-Src.

Overexpression of c-Src and HER family members in human breast carcinoma cell lines. A panel of thirteen human breast carcinoma cell lines was analyzed by Western blotting for levels of c-Src and HER family members (Figure 1). Scanning densitometry of autoradiographs of the immunoblots is shown in Table II. Nine of these cell lines expressed c-Src greater than two-fold above the immortalized, non-tumorigenic human breast epithelial cell line, HS578Bst. All but one of the cell lines overexpressed HER1, HER2 or HER3, in various combinations. Within any given cell line, however, overexpression of HER1 and HER2 appeared to be mutually exclusive. This finding agrees with published literature, which indicates that HER2 is typically expressed in earlier stage breast carcinomas (De Potter *et al.*, 1990; Gusterson *et al.*, 1988; Gusterson *et al.*, 1988b; Maguire *et al.*, 1992; Ramachandra *et al.*, 1990; van de Vijver *et al.*, 1988), while HER1 is expressed in later, more aggressive tumors (Battaglia *et al.*, 1988; Sainsbury *et al.*, 1987; Toi *et al.*, 1991). In addition, HER2 and HER3 were found to be co-overexpressed in the majority of cell lines, thereby enhancing the probability of formation of the preferred, more tumorigenic HER2/HER3 heterodimer. Levels of HER4 could not be assessed with confidence, due to the unavailability of reliable immunoblotting antibodies.

HRG-enhanced soft agar colony formation. In human breast tumor cell lines, HRG treatment has been demonstrated to increase tyrosine phosphorylation of many cellular proteins that are involved in mitogenic signaling pathways (Belsches-Jablonski, unpublished observations; Carraway *et al.*, 1995). Substrates of HER2 and HER3 include SHC (Segatto *et al.*, 1993; Xie *et al.*, 1995), p21^{ras}GAP, PLCγ (phospholipase Cγ)(Fazioli *et al.*, 1991), paxillin (Romano *et al.*, 1994), and PI-3 kinase (Sepp-Lorenzino *et al.*, 1996). It was therefore of interest to determine if HRG treatment affected the mitogenic or tumorigenic properties of these cell lines. To investigate this question, we chose a subset of the cell lines described in Table II, based on their varying expression of HER family members and c-Src. Three cell lines (MDA-MB-361, MDA-

MB-453, UACC-812) were selected because they co-overexpressed moderate to high levels of HER2 and HER3. Of these, only MDA-MB-361 overexpressed c-Src. MCF-7, MDA-MB-468 and SK-BR3 cells were selected because of their generally low levels of HER2/HER3 and moderate to high levels of c-Src. Figure 2 shows that only the cell lines that co-overexpressed HER2 and HER3 (MDA-MB-361, MDA-MB-453, UACC-812) responded to HRG in a soft agar colony formation assay. HRG treatment potentiated anchorage-independent growth of these cell lines two to three-fold over that of their growth in 10% serum (Fig. 2, inset), while having no significant effect on serum-induced colony formation of MCF7, MDA-MB-468, or SK-BR3 cell lines. These data suggest that HRG-induced heterodimerization of HER2 and HER3 or HER4 promoted a ligand-dependent increase in anchorage-independent growth, a classic indicator of tumorigenicity. Treatment of cells with the Src kinase family inhibitor, PP1, partially or completely inhibited HRG-enhanced soft agar growth in MDA-MB-361, MDA-MB-453, and UACC-812 cells, as well as variably reducing growth in serum alone in all cell lines.

HRG-potentiated anchorage-dependent growth. Because HRG treatment led to an increase in anchorage-independent growth of the three cell lines that co-overexpressed HER2 and HER3, we wished to determine if HRG also affected anchorage-dependent growth of these or the control cell lines. An MTT assay was used to follow cell number as a function of incubation time in the various treatment conditions. No effect of HRG on the cell number of any of the cell lines was observed in 10% serum, even when assessed up to two weeks after plating. However, Figure 3 shows that HRG treatment resulted in a small, but significant increase in cell number in five of the cell lines (MDA-MB-361, UACC-812, MCF7, MDA-MB-468, and SK-BR3) tested, when they were maintained in reduced serum (0.5%) over a five day period (Panels A, C, D, E and F; compare "day 5" to "day 5+H"). Only one cell line (MDA-MB-453) did not respond to HRG in this assay. Interestingly, the effect of HRG on cell numbers did not correlate with the levels of HER2, HER3, or c-Src in the various cell lines.

PP1 treatment either inhibited the growth or reduced the viability of all cell lines maintained in low serum alone (Fig. 3; compare "day 5 + P" to "day 5" in each panel), but the effect of PP1 on cells treated with HRG varied from cell line to cell line. HRG-treated MDA-MB-361 and MCF7 lines were only slightly reduced in cell number by PP1 treatment when compared to cell numbers following incubation with HRG in the presence of 0.5% serum vs. those in the presence of 0.5% serum alone (Fig.3, Panels A and D; compare "day 5 + H + P" to "day 5 + H" and "day 5"), while HRG-treated SK-BR3 was partially reduced (Panel F), and UACC-812 and MDA-MB-468 were completely reduced to levels equal to or below that of cells maintained in 0.5% serum alone (Panels C and F). Again, the effect of PP1 on HRG-modulated cell number did not correlate with the levels of HER2, HER3, or c-Src in the various cell lines. These results suggest that c-Src and HRG cooperate in the majority of breast cancer cell lines tested, but the extent of this cooperation varies considerably from cell line to cell line.

To determine if PP1 was indeed inhibiting c-Src in the breast cancer cells, c-Src was immunoprecipitated from MDA-MB-361 cells, preincubated with varying concentrations of PP1 (ranging from 0.1μM to 10μM) for 15 minutes, and its activity was assessed in an *in vitro* immune complex kinase assay, using γ-[^{32}P]-ATP. The auto-phosphorylation activity of c-Src were reduced by PP1 in a dose- and time-dependent manner (data not shown). At the highest concentration (10μM), PP1 completely inhibited c-Src catalytic activity in a 10 min incubation. Together these results verified that PP1 functioned as expected.

To further assess the role of c-Src and its family members on the growth and viability of breast cancer cells, we attempted to generate stable transfectants of MDA-MB-361 cells that expressed a dominant negative, kinase-deficient form of chicken c-Src (V430A) (Wilson, *et al.*, MCB, 1989) that had been subcloned into the pcDNA3 mammalian expression vector (Invitrogen and Tice *et al.*, 1998a, in preparation). Transfected cells were selected for neomycin resistance, and early, antibiotic-resistant populations of cells were shown to express high levels of the variant form of c-Src by Western blotting with the chicken c-Src-specific MAb, EC10. However, no long-term stable clones could be obtained from this population, even after repeated efforts (data not shown). These data suggest a striking dependence on c-Src kinase activity for long-term survival and growth of these cells, even in full serum conditions. Not all human breast cancer cell lines behaved as MDA-MB-361 cells, in that stable clones expressing kinase-defective c-Src could be obtained from them. Examples of such cell lines include MDA-MB-468 and MDA-MB-231. Similar to MDA-MB-361 cells, however, their tumorigenic properties were impaired (Tice *et al.*, 1998b, in preparation).

Heregulin reverses the apoptotic effect of PP1 in reduced serum. Because HRG treatment appeared not only to increase cell number in the majority of breast tumor cell lines in the presence of reduced serum alone but also in the presence of PP1, we examined one of the cell lines (MDA-MB-361) for possible evidence of an anti-apoptotic effect of HRG. Cells were cultured for three days in the presence or absence of HRG and/or PP1, alone and in combination with wortmannin, an inhibitor of the anti-apoptotic or survival activity of PI_3 kinase (Wymann *et al.*, 1996), and then analyzed for evidence of apoptosis, using DAPI-staining of DNA to detect abnormally shaped nuclei (Villaneuva *et al.*, 1984). Figure 4, Panel A shows that PP1 treatment of MDA-MB-361 cells induced apoptosis in 10-15% of the cells (vs. ~3% in 0.5% serum alone) and that PP1-induced apoptosis was completely reversed by HRG. UV-induced apoptosis was used as a positive control (>85%). Interestingly, HRG action was only partially wortmannin-sensitive, suggesting HRG functions via PI_3 kinase-dependent and -independent mechanisms to promote cell survival. Apoptosis results were verified with the TUNEL assay (data not shown).

Physical association of c-Src with HER2 in human breast carcinoma cell lines. Given the body of existing evidence for physical interactions between c-Src and HER family members (especially HER1 and HER2 (Luttrell *et al.*, 1994; Muthuswamy and Muller, 1995) and the apparent functional cooperativity between c-Src and HRG in promoting growth and survival of breast tumor cells (as described above), we analyzed our panel of human breast carcinoma cell lines by co-immunoprecipitation and Western blotting for evidence of *in vivo* association between c-Src and HER2 to determine if there was a correlation between c-Src/HER2 association and HRG-promoted growth and/or survival. Figure 5, Panel A depicts results from an analysis of four of the fourteen cell lines tested (MDA-MB-361, MDA-MB-453, MCF7, MDA-MB-468), and the outcome of the entire analysis is summarized in Table II. Only three cell lines of the entire panel exhibited an *in vivo* association between c-Src and HER2 (MDA-MB-361, MDA-MB-453, UACC-812), although nine of the fourteen overexpressed HER2 and seven of the nine also overexpressed HER3. The presence of HER2 in c-Src immunoprecipitates was reproducibly seen, but the presence of c-Src in HER-2 immunoprecipitates was more difficult to detect. The reason for this is unclear. Association was observed under conditions of serum deprivation and was not altered by HRG stimulation of the cells (200ng/ml for 5 min) (Panel A). The presence of constitutive complexes between c-Src and HER2 in the breast cancer cell lines suggests that a growth factor autocrine loop(s) may be promoting c-Src/HER2 interactions.

Only one of the three cell lines that showed association overexpressed c-Src (MDA-MB-361), indicating that association was not dependent on overexpression of c-Src. However, HER2 and HER3 were overexpressed in all three cell lines. These results are in striking contrast to our findings with regard to c-Src/HER1 interactions, where overexpression of both molecules was necessary in order to detect physical association between the two. This requirement was noted in both a mouse fibroblast model and in human breast cancer cells (Maa, *et al.*, 1995; Biscardi *et al.*, 1998). Interestingly, as shown in Table II and Figure 2, the ability of HRG to stimulate anchorage-independent growth in the six cell lines tested correlated with the presence of an *in vivo* complex between c-Src and HER2, suggesting that physical association between c-Src and HER2 may contribute to HRG-promoted anchorage-independent growth. However, such a correlation was not seen between the functional involvement of c-Src in HRG-stimulated anchorage-dependent growth and complex formation, suggesting that alternative mechanisms of cooperativity are involved in this process (Table III and below).

The low level of c-Src in some of the cell lines made it difficult to convincingly demonstrate that c-Src was indeed in the HER2/c-Src complex. However, despite the fact that c-Src could not be detected by Western blotting in whole-cell lysates (Fig. 1 and Table II) or in c-Src-immunoprecipitations using 2-17 c-Src-specific MAb and 500 μg lysate (Fig. 5A with the MDA-MB-453 cell line), Figure 5, Panel B shows that very low levels of c-Src could be detected in the MDA-MB-453 cell line when a mixture of Src antibodies to the N-terminus (2-17 Mab) and the SH3 domain (GD11) and 2500 μg lysate were used. Similar results were seen with the UACC-812 cell line. Thus c-Src was present in the complexes precipitated from the MDA-MB-453 and UACC-812 cell lines, but in low concentrations. These findings suggest that stoichiometric interactions between HER2 and c-Src are not necessary for the functional effects of c-Src on HRG-induced responses or, alternatively, that the complexes do not play a major role in the biological processes regulated by HRG. This issue will be discussed further.

Figure 5, Panel A shows some non-specific association with the negative control antibody of a protein from MDA-MB-361 cells that migrated similarly (but not always identically) to HER2. The levels of this protein in control immunoprecipitates varied from experiment to experiment but were consistently less than that seen with the c-Src antibody under the same conditions of immunoprecipitation. To address this issue further, a panel of c-Src-specific antibodies and negative control antibodies was used to determine specificity of the interaction. Figure 5, Panel C shows that co-immunoprecipitation of HER2 with c-Src was specific, as evidenced by the presence of HER2 in complexes with c-Src, when multiple c-Src antibodies, directed to different regions of the molecule, were used in independent assays, and the absence of or significantly reduced levels of HER2 (or a similarly migrating protein) in several different negative antibody control precipitates.

Association of c-Src with HER2 in human breast tumor samples. Because of the questions raised above about the functional significance of the physical association between c-Src and HER2 and since HER family members and c-Src have been implicated in the formation of human breast tumors, we wished to determine whether c-Src/HER2 complexes existed in human breast carcinoma tissue or in normal breast tissue, and if so, how frequently. A panel of thirteen tumor tissues, obtained from the University of Virginia and University of Michigan tumor banks, were analyzed by immunoprecipitation and Western blotting with antibodies to HER2 and c-Src, in a fashion similar to that used for the breast tumor cell lines. Figure 6 shows examples of this

analysis. Tumors UVA156 and UVA263 were scored as positive for c-Src/HER2 complex formation (above the level of the negative antibody control), whereas MichN1 was scored as negative. In UVA263 reciprocal co-precipitation between c-Src and HER2 was observed. In addition, HER2/HER1 (UVA156 and UVA263) and HER2/HER3 (UVA156) complexes could be seen, indicating constitutive heterodimerization between HER family members in these tumors, perhaps due to chronic autocrine stimulation. Normal breast tissue contained detectable levels of c-Src but undetectable levels of HER 2, and no association between HER2 and c-Src, HER1, or HER3 was observed. Table III summarizes the results of such an analysis for all thirteen tumor samples and describes tumor type and grade, estrogen receptor (ER) status and lymph node involvement, as well as relative levels of c-Src and HER2 protein and the presence of a stable complex between c-Src and HER2 for each tumor. In all, stable association between c-Src and HER2 was seen in three out of thirteen samples, the same frequency as seen for the breast tumor cell lines. In contrast to the cell lines, however, c-Src/HER2 complexes were seen only in tissues that overexpressed both HER2 and c-Src. Complex formation between c-Src and HER2 has been demonstrated in mammary tumors in transgenic mice (Muthuswamy *et al.*, 1994), but this is the first demonstration of an *in vivo* association in human breast carcinoma samples.

Signal transduction of SHC and MAPK in human breast carcinoma cell lines. In attempts to discern the signal transduction pathways which may be activated by HRG when the HER2/c-Src complex is present, we have examined four of the breast carcinoma cell lines for phosphorylation of SHC and MAPK in the presence of HRG (Figures 7 and 8). In one of the cell lines which overexpress HER2, and exhibits heterocomplex formation between HER2 and c-Src (MDA-MB-453), SHC is constitutively phosphorylated. This finding is consistent with the constitutive phosphorylation of MAPK in this cell line. However, MAPK phosphorylation is inducible by HRG, suggesting a pathway other than SHC may be contributing to the cumulative phosphorylation of MAPK, or perhaps different residues of MAPK become phosphorylated by HRG stimulation. In another cell line which shows a heterocomplex between HER2 and c-Src, MDA-MB-361, where there is low constitutive phosphorylation of both SHC and MAPK, phosphorylation of both molecules is inducible by HRG. Taken together, these data suggest that there are multiple pathways to activate mitogenesis via MAPK in these cell lines, mediated by multiple HER family members.

Additional experiments were performed to identify novel substrates of HER2, including immunoprecipitations of paxillin, cortactin, and CHK. None of these molecules were shown to immunoprecipitate with HER2 in a variety of cell lines under our conditions.

DISCUSSION

Both HER family members and c-Src are overexpressed at high frequency in human breast cancers (Slamon *et al.*, 1987; Slamon *et al.*, 1989; Szollosi *et al.*, 1995; Juhl *et al.*, 1997; this report) and have been implicated in the genesis and progression of the disease. Amplification/overexpression of HER2, especially, has been associated with a poor patient prognosis (Slamon *et al.*, 1987; Slamon *et al.*, 1989). Recent studies from our laboratory have demonstrated a synergistic interaction between c-Src and HER1 in tumor formation (Maa *et al.*, 1995; Biscardi *et al.*, 1998, submitted), suggesting that similar interactions may exist between c-Src and other HER family members. Here we describe the results of experiments designed to

address this issue in the context of human breast tumor cell lines and tumor tissue. In these studies, tumor cell lines were treated with ligands that activate HER3/HER4 and induce homo- and hetero-dimerization with HER2, as well as with an inhibitor of Src family kinases, PP1, in order to assess the contribution of each family of kinases and of their potential interaction to the growth of the cells. We found that PP1 is a potent inhibitor of proliferation and/or survival of all cell lines tested (six), when cultured under various conditions of anchorage-independency (Fig. 2) or -dependency (Fig. 3), in the presence of full serum (10%; Fig. 2) or reduced serum (0.5%; Fig. 3). We interpret these results to mean that c-Src and other Src family members that may be expressed in the various cell lines participate in critical pathways that maintain viability and promote growth of cells under serum maintenance. Heregulin, on the other hand, is capable of partially reversing the growth inhibitory or apoptotic effects of PP1 (Fig. 4), suggesting that HRG can activate survival and growth pathways that are independent of c-Src and its relatives. The extent to which HRG succeeds at this task varies from cell line to cell line (almost completely in MDA-MB-361 and MCF7, partially in UACC-812, and poorly in MDA-MB-453; Figs. 2 and 3), intimating that HRG and c-Src can overlap in their signaling pathways and that the degree to which this overlap occurs is dependent on the cell line and the array of signaling pathways that are activated in each. Thus, HRG has the capability of functioning via Src family member-independent and dependent mechanisms.

One mechanism by which HRG may be dependent on Src family members is through physical association of HER2 with c-Src. Indeed, in 23% of cell lines and tumor tissues tested (3/13 in each case), a stable and specific association between the two kinases was observed. Interestingly, this association was insensitive to exogenous HRG, but its constitutive nature suggests that it may be formed in response to chronic autocrine stimulation. The ability to detect c-Src/HER2 complexes in the breast tumor cell lines correlated with the ability of HRG to promote colony formation in soft agar in these same cell lines, suggesting that the complex may participate in anchorage-independent growth. Involvement of c-Src in such a process is consistent with its documented roles in regulating focal adhesion formation (cell-substratum interactions) and inter-cellular interactions via cadherins (reviewed in Parsons and Parsons, 1997; Malik and Parsons, 1996).

However, the ability to detect c-Src/HER2 complexes in the various breast tumor cell lines did not correlate with the growth-promoting and/or the anti-apoptotic effects of HRG, when the various cell lines were assayed for anchorage-dependent growth responses in reduced serum. These findings suggest that Src family members and HER2 interact via other mechanisms, such as participating in the same pathway at different points or by acting in different pathways that converge downstream of the respective points of action. In favor of the latter is our inability to detect c-Src activation in response to HRG treatment (data not shown), a finding that suggests that c-Src does not lie directly downstream of HRG receptors.

Alternatively, HRG could be activating Src family members other than c-Src. This is a possibility, given that other members, such as Fyn and Yes, have been found to be activated in certain types of human tumors, such as colon (Pena *et al.*, 1995; Rosen *et al.*, 1986; Bolen *et al.*, 1987a, 1987b; Cartwright *et al.*, 1989) and lung (Mazurenko, 1991a, 1991b; Cook *et al.*, 1993). However, these family members are much less frequently activated or overexpressed in human breast cancer cells or tissues than is c-Src, suggesting that c-Src should be the focus of our attention.

Despite similarities between the structure of HER1 and HER2, and their involvement in the genesis of breast cancer and other human tumors, there are striking differences between immune complexes of c-Src with HER1 versus HER2. In both our model system and in a subset of human breast tumor cell lines, physical association of c-Src with HER1 is EGF-dependent, and occurs only in the presence of overexpression of both c-Src and HER1 (Maa *et al.,* 1995; Biscardi *et al.,* 1998). In contrast, the formation of HER2/c-Src complexes is constitutive and insensitive to HRG treatment, suggesting autocrine loops at work. Given the many ligands that have been shown to bind to HER family members, it may not be surprising that autocrine production of ligands allows constitutive activation of the receptors. Blotting of unstimulated whole cell lysates with an anti-phosphotyrosine antibody demonstrates phosphorylated protein in the 170-185kDa range in each cell line which exhibits the c-Src/HER2 complex (data not shown). Treatment of these cell lines with EGF or HRG does not increase the overall amount of phosphotyrosine signal.

In addition, overexpression of neither HER2 nor c-Src was required for physical association in the cell lines, such as in MDA-MB-453, although low levels of each molecule could be detected (Figure 5B). Perhaps a complex of c-Src, HER2 and HER3 in this cell line may be involved. In tumor tissue, however, complex formation between HER2 and c-Src only occurred when both kinases were overexpressed. This finding may indicate differences in signaling in *in vivo* tumors, versus long term *in vitro* cell cultures.

More information about the mechanism of action between HER2 and c-Src can be obtained through studies of potentially novel sites of phosphorylation on HER2 by c-Src in the breast cancer cell lines, as well as expression of wildtype and mutant forms of HER2 in the C3H10T1/2 mouse fibroblast model system in Specific Aim I.

Progress towards Specific Aims.

Specific Aim I. Derive stable cells lines overexpressing c-Src or wild-type HER2 alone or in combination and examine them for growth, morphology and tumorigenic properties. Other transformants to be assayed include activated HER2, kinase-mutant HER2, and a chimeric receptor containing the extracellular domain of HER1, and the transmembrane and intracellular domains of HER2.

Moderate progress has been achieved for this specific aim, and new methods of transfection were successful in obtaining clones that exhibit morphological differences. Because it has been difficult to assess the amount of overexpression of the HER2 constructs by Western blotting, future attempts by other laboratory personnel will utilize newly-constructed HER2 cDNA constructs which are known to express well in mouse fibroblast cell lines.

Specific Aim II. Screen a panel of human breast carcinoma cell lines for overexpression of c-Src or HER2, or both. In cell lines overexpressing only one of these, overexpress the missing partner and assay for effects on tumorigenicity.

Screening of this panel is complete. Assays of tumorigenicity for the panel of six wildtype carcinoma cell lines used in the study yielded no tumors in nude mice, but studies in soft agar were successful to assess tumorigenicity (Figure 2). No studies of overexpression of missing partners in these cell lines has been completed.

Specific Aim III. Examine derived cell lines overexpressing c-Src and HER2 as well as breast carcinoma cell lines for *in vivo* association of c-Src with HER2. If *in vivo* association is evident, examine both c-Src and HER2 immunoprecipitated in the complex for novel sites of phosphorylation, and assay for differences in the phosphorylation state of downstream substrates of HER2 and c-Src.

Assays for c-Src/HER2 complex formation in breast carcinoma cell lines are complete, as well as assays for changes in phosphorylation of downstream substrates. Novel sites of phosphorylation for the HER2 receptor while in complex with c-Src are not complete.

Specific Aim IV. Derive stable cell lines overexpressing wild-type HER1 and HER2 simultaneously, with and without overexpression of c-Src, and assay for changes in growth, morphology and tumorigenicity. Comparisons will be made between these cell lines and those derived to overexpress HER2 alone, with or without overexpressing c-Src.

This specific aim has not been completed.

CONCLUSIONS

- HER family members as well as c-Src are overexpressed in the majority of human breast tumor cell lines and breast tumors tested (total of 27).

- In 3 of 14 breast tumor cell lines and in 3 of 13 human breast tumors, HER2 and c-Src are physically associated in an immune complex. However, in contrast to the HER1/Src model system, overexpression of neither HER2 or c-Src is required for physical association.

- The three breast tumor cell lines exhibiting the HER2/c-Src complex (MDA-MB-361, MDA-MB-453 and UACC-812) show an increase in growth rate in response to HRG, as assayed by MTT assay. This effect can by ablated by the Src family kinase inhibitor, PP1. Two cell lines, SK-BR3 and MCF7, which do not exhibit the HER2/c-Src complex, also show an increased growth rate in the presence of HRG. This effect cannot be ablated by PP1 in MCF7 cells, suggesting another mechanism for the HRG-dependent effect.

- In addition, MDA-MB-361 and UACC-812 show increased number of colonies formed in soft agar in the presence of HRG. Taken together with the MTT growth assays, these data suggest a correlation between HER2/c-Src association and HRG-dependent increased growth rates and tumorigenicity.

- One of the 3 cell lines (MDA-MB-361) exhibiting the HER2/c-Src complex shows inducible SHC phosphorylation in response to HRG, while another (MDA-MB-453) shows constitutive phosphorylation of SHC, and inducibility by HRG. These data indicate that there is no correlation between HER2/c-Src association and constitutive SHC phosphorylation.

- MAPK shows low basal levels and activation in all cell lines (to varying degrees) in response to HRG. In MDA-MB-361 and MCF7 cells, activation of MAPK correlates with SHC tyrosine phosphorylation.

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APPENDIX

FIGURE LEGENDS

Figure 1. Overexpression of HER family members and c-Src in human breast carcinoma cell lines. Lysates from indicated cell lines were prepared in RIPA buffer, and 100μg protein was loaded per well in a 7% polyacrylamide gel. Western blots were probed with antibodies to c-Src (2-17); HER1 (3A4A); HER2 (Santa Cruz SC-284); and HER3 (Santa Cruz SC-285). Primary antibodies were visualized by ^{125}I-protein A.

Figure 2. Effects of HRG and the Src kinase family inhibitor, PP1, on anchorage-independent growth of human breast tumor cells. MDA-MB-361, MDA-MB-453, UACC-812, MCF7, MDA-MB-468 and SK-BR3 cells were plated in soft agar and incubated at 37°C in a humidified, 5% CO_2 atmosphere for two weeks in DMEM containing the following: 10% serum alone; 10% serum + 40 ng/ml HRG-α; 10% serum + 40 ng/ml HRG-α + 10 μM PP1; or 10% serum + 10 μM PP1. Media with additives was replenished every 4 days. Colonies were stained, counted, and expressed as the mean colony number ± SEM of three independent experiments, each performed in triplicate. Inset depicts fold difference of HRG treatment over non-stimulated controls. * = $p \leq 0.05$, comparing 10% serum vs. 10% serum + HRG. # = $p \leq 0.05$, comparing 10% serum + HRG + PP1 vs. 10% serum + PP1.

Figure 3. Effects of HRG and PP1 on anchorage-dependent, reduced-serum growth and survival of human breast cancer cells. MDA-MB-361 (**Panel A**), MDA-MB-453 (**Panel B**), UACC-812 (**Panel C**), MCF7 (**Panel D**), MDA-MB-468 (**Panel E**) and SK-BR3 (**Panel F**) cells were plated in DMEM with the following additives: 0.5% serum alone; 0.5% serum + 40 ng/ml HRG-α or -β; 0.5% serum + HRG + 10 μM PP1; or 0.5% serum + 10 μM PP1 and incubated for 5 days, with replenishment of media containing the appropriate additives at day 2.5. Cell number at Days 0 and 5 was determined by the MTT assay and is expressed as mean fold-change ± SEM relative to Day 0. Day 0 density was determined 12 hours after plating. Results are pooled from 4 independent experiments, each performed in triplicate. * = significantly different than day 0. # = significantly different than day 5, 0.5% serum. ψ = significantly different than day 5, 0.5% serum + HRG. p-values ≤ 0.05

Figure 4. Anti-apoptotic effects of HRG and Src family kinases in human breast tumor cell line MDA-MB-361. Cells were plated onto coverslips (5 X 10^4 cells/coverslip) and grown in one of the following media: 0.5% serum alone; 0.5% serum + 40 ng/ml HRG-α; 0.5% serum + 10 μM PP1; 0.5% serum + HRG + 10 μM PP1; 0.5% serum + 40 ng/ml HRG-α + 4nM wortmannin; 0.5% serum + 10 μM PP1 + 4nM wortmannin; or 0.5% serum + HRG + 10 μM PP1 + 4nM wortmannin, and incubated for 3 days. After that time, cells were fixed in 4% paraformaldehyde and permeabilized, and nuclei stained for DNA with DAPI. At least 400 cells per treatment were quantitated for apoptotic nuclei in two separate experiments. A 2-minute UV-B treatment and 48 hour recovery in serum-free media was used as a positive control for apoptosis. * = significantly different than heregulin (H) treatment, in the absence or presence of

wortmannin. # = significantly different than PP1 (P) treatment, in the absence or presence of wortmannin.

Figure 5. Physical association between c-Src and HER2 in human breast carcinoma cell lines. Panel A: MDA-MB-361, MDA-MB-453, MCF7 and MDA-MB-468 cells were serum-starved for 17 hr. and either stimulated with 200 ng/ml HRG-α (+) or incubated in fresh serum-free media (-) for 5 min. Cells were then lysed, and 500 μg lysate protein from each cell line and treatment protocol was incubated with either α-Src (S) (2-17 Mab), α-HER2 (H2) or mouse IgG. Precipitated proteins were separated by SDS-PAGE and transferred to a nylon membrane. The upper half of the membrane was probed with α-HER2 primary antibody, and the lower half was probed with α-Src (2-17) primary antibody. Both primary antibodies were visualized by ^{125}I-protein A. **Panel B.** MDA-MB-361 and MDA-MB-453 cells were treated as in (**A**), except that 2.5 mg lysate protein and a mixture of 2-17 and GD11 c-Src-specific Mabs was used for the immunoprecipitation. The nylon membrane was probed with 2-17 Mab. Primary antibody was visualized by ^{125}I-protein A. **Panel C.** Lysates from unstimulated MDA-MB-361 cells were prepared as in (**A**), and 1.0 mg lysate protein was immunoprecipitated with each of four different antibodies to c-Src (see Materials and Methods), as well as mouse α-rabbit (MAR) or mouse IgG as negative antibody controls. Immunoblots were prepared as in (**A**).

Figure 6. Presence of endogenous c-Src/HER2 complexes in human breast tumor tissue. Tumor lysates were homogenized, lysed in RIPA detergent buffer, and 250 μg lysate protein was incubated with either α-Src (S), α-HER1 (H1), α-HER2 (H2), or α-HER3 (H3). Precipitated proteins were separated on a 7% polyacrylamide gel and transferred to nylon membrane. The upper half was probed with α-HER2, and the lower half probed with 2-17 Mab. Both primary antibodies were visualized by ^{125}I-protein A. (-) indicates mouse α-rabbit IgG as a negative control for immunoprecipitation.

Figure 7. Constitutive and heregulin-inducible tyrosine phosphorylation of SHC in human breast carcinoma cell lines. Cultures of MDA-MB-361, MDA-MB-453, MCF7 and MDA-MB-468 cells were serum-starved overnight, and stimulated for five minutes with heregulin-α (200ng/ml). Lysates were prepared in RIPA detergent buffer, and immunoprecipitated with α-SHC antibody. IPs were separated on a 7% SDS-polyacrylamide gel, transferred onto nylon membrane and immunoblotted with α-phosphotyrosine (p-Tyr) antibody or α-SHC antibody. The primary antibody was recognized by ^{125}I-protein A.

Figure 8. Activation of MAP kinase by EGF and heregulin-α in human breast carcinoma cell lines. Cell cultures were serum-starved overnight, and stimulated for five minutes with either EGF (100ng/ml) (E) or heregulin-α (200ng/ml) (H). Lysates were prepared in RIPA detergent buffer and 100μg protein loaded per well on a 7% SDS-polyacrylamide gel, transferred onto nylon membrane and immunoblotted with either α-phospho-MAPK antibody or α-MAPK (B3B9). The primary antibody was recognized by ^{125}I-protein A.

Table I.
Development of stable transfectant cell lines.

Constructs Needed:	Control Background:	c-Src Overexpressor:
wildtype HER2	7 (3X)[1]	15 (unknown)
oncogenic HER2 (V659E)	4 (5X)	
HER1/HER2 chimera		
kinase-deficient HER2 (L753R)		3 (24X)
phosphorylation-site mutant HER2 (Y877F)	3 (6X)	

[1]Indicates number of clones obtained and (fold overexpression).

Table I. Development of stable transfectant cell lines. Stable clones of C3H10T1/2 mouse fibroblasts which overexpress wildtype or mutant HER2 protein are listed, with the relative levels of expression above parental 10T1/2 cells shown in parentheses. C-Src overexpressors (5Hd47) cells express c-Src protein approximately 25-fold over endogenous 10T1/2 levels (Luttrell *et al.*, 1988, Wilson *et al.*, 1989).

Table II
Human Breast Carcinoma Cell Lines

Relative levels of HER family members and c-Src

					Association of c-Src with:		
cell line	HER1	HER2	HER3	c-Src	ER	HER1	HER2
MDA-MB175	*	2.2	6.1	9.9	+	-	-
UACC-893	*	72.8	6.7	*	-	ND	ND
UACC-812	*	50.8	6.1	*	-	-	+
SK-BR-3	12.6	2.5	*	19.4	-	+	-
MDA-MB361	*	12.8	10.9	37.4	+	-	+
MDA-MB453	*	1.5	6.1	*	-	-	+
MDA-MB468	39.5	*	*	4.9	-	+	-
ZR75-B	*	*	1.1	6.2	+	-	-
BT-474	*	61.8	26.4	*	+	-	-
BT-549	6.9	*	*	13.4	-	+	-
MDA-MB231	7.6	1.1	1.0	2.9	-	+	-
BT-20	3.4	1.9	5	13.5	-	+	-
MCF-7	1	1	2.3	6.4	+	-	-
Hs578Bst[1]	1	*	*	1	+	-	-

(*) = below limits of detection ND in table indicates no data.

Table I. Relative levels of HER family members and c-Src in human breast carcinoma cell lines. Autoradiographs from Figure 1 were analyzed by densitometry and compared to Hs578Bst[1], an immortalized normal human mammary epithelial cell line. The lowest detectable signal on each blot was set as 1.0, and fold difference of the cell lines determined. An * indicates a signal was below the limits of detection by Western blotting. Also included in the table are estrogen receptor (ER) status of the cell lines, as determined by Western blotting (Biscardi *et al.*, 1998), and physical association between c-Src and either HER1 or HER2, as determined by immunoprecipitation with monoclonal antibodies to c-Src.

Table III

Summary of heregulin and PP1 effects on soft agar colony formation and cell number

cell line	Src/HER2 complex	HRG Effect on soft agar growth	HRG Effect on cell number	PP1 Effect on HRG+0.5% serum cell number	PP1 Effect on 0.5% serum cell number
MDA-MB361	+	+	+	-	+
MDA-MB453	+	+	-	+	+
UACC-812	+	+	+	+	+
MCF-7	-	-	+	-	+
MDA-MB468	-	-	-	+	+
SK-BR-3	-	-	+	+	+

Table III. Statistically significant effects of heregulin or PP1 are charted with correlation of c-Src/HER2 complex formation.

Table IV

Summary of Human Breast Tumors Tested for Physical Association of HER2 and c-Src

Tumor	Tumor Type	ER	Lymph Node	Src/HER2 Association	RELATIVE LEVELS c-Src	HER2
UVA103	IVDC[1], grade 3/3	-	-	-	ND[6]	ND
UVA156	IDC[2], grade 2/3	-	+	+	+++	+++
UVA226	IDA[3], grade 2/3	+	ND	+	++	+++
UVA263	IDC, grade 3/3	ND	+	+	+++	+++++
UVA387	ILC[4]	+	ND	-	+	*[7]
UVA399	IDC, grade 3/3	-	ND	-	+	+++
UVA454	IDC, grade 3/3	-	+	-	++	++
UVA616	IVDC, grade 3/3	-	+	-	ND	ND
MICHN1	medullary, grade 3/3		ND	-	+	+
MICHP1	#[5]	ND	ND	-	*	++
MICHP2	#	ND	ND	-	*	+
MICHP3	#	ND	ND	-	*	+
MICHP4	#	ND	ND	-	*	+

n = 13 3/13 = 23%

[1]IVDC = invasive ductal carcinoma
[2]IDC = infiltrating ductal carcinoma
[3]IDA = infiltrating ductal adenocarcinoma
[4]ILC = infiltrating lobular carcinoma
[5]# = data currently not available
[6]ND = no data
[7]* = below limits of detection

Table IV. Summary of human breast tumors tested for physical association of HER2 and c-Src. Human breast tumor samples obtained from the UVA Tissue Procurement Facility and the University of Michigan Tumor Bank were analyzed by immunoprecipitation and Western blotting for physical association between HER2 and c-Src. Tumor type and grade, ER and lymph node status, and relative levels of HER2 and c-Src protein are listed for each tumor.

Human Breast Carcinoma Cell Lines Overexpress HER Family Members and c-Src Protein

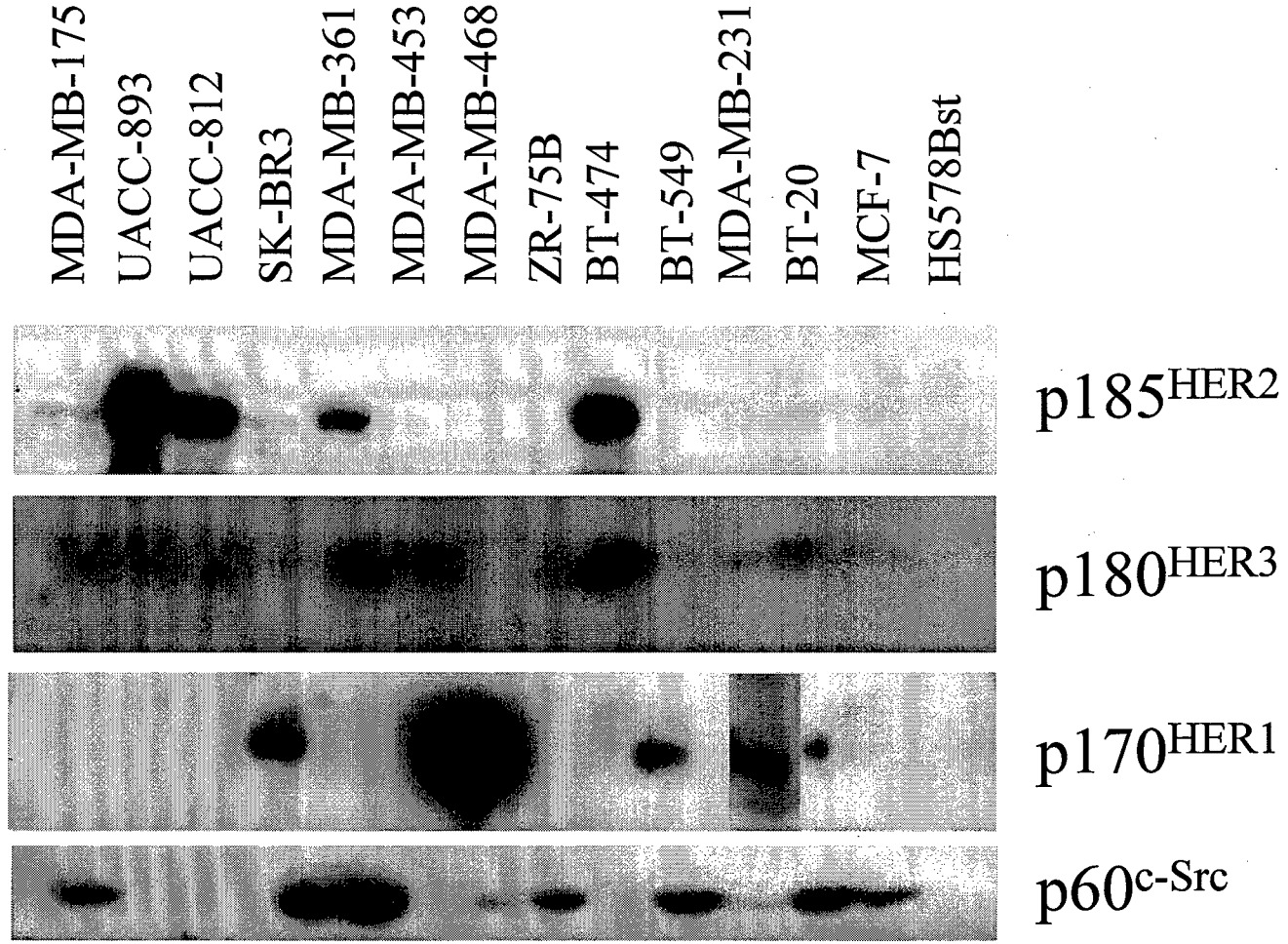


FIGURE 1

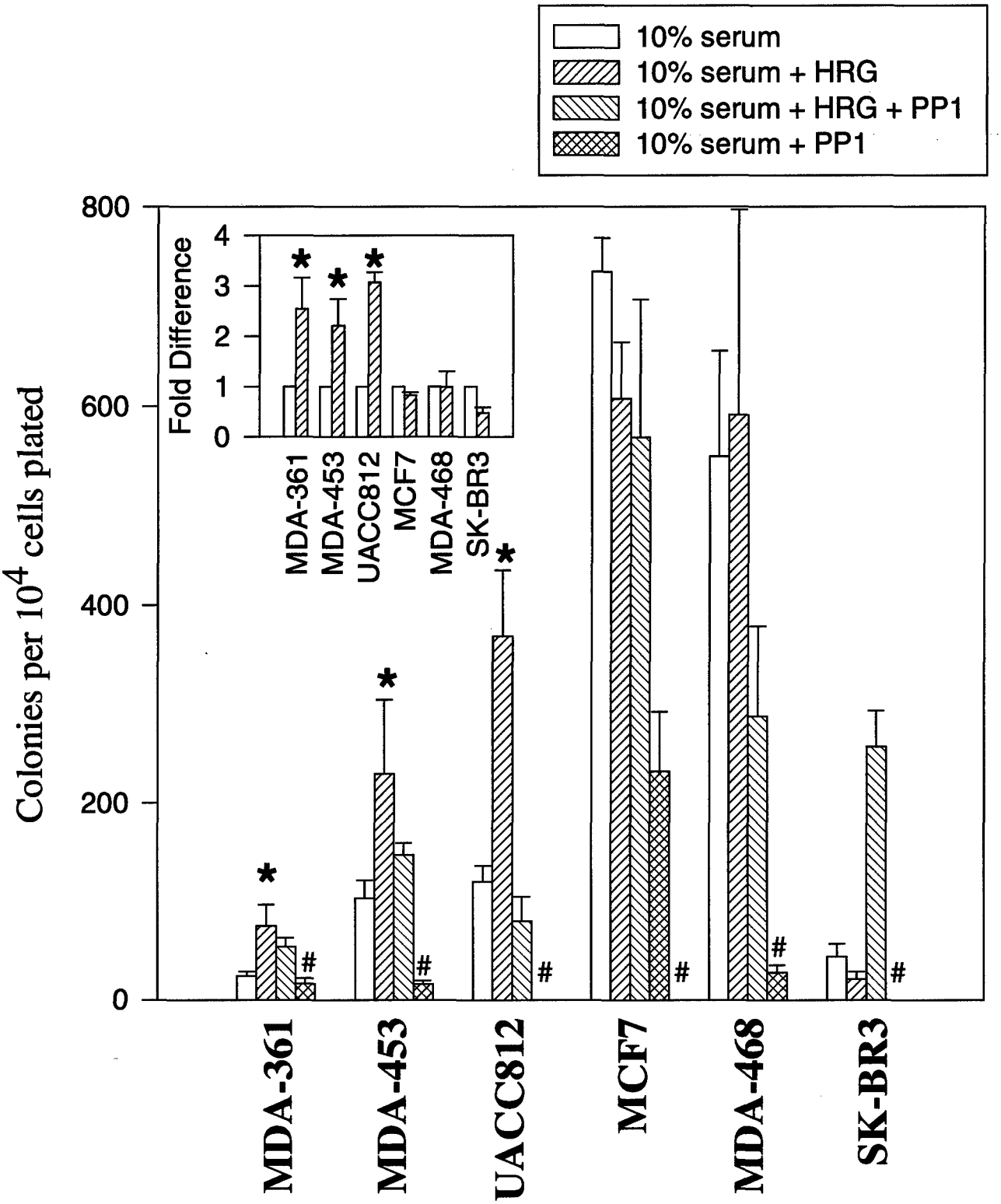

10% serum
10% serum + HRG
10% serum + HRG + PP1
10% serum + PP1
Colonies per 10^4 cells plated
800
600
400
200
0
Fold Difference
4
3
2
1
0
MDA-361
MDA-453
UACC812
MCF7
MDA-468
SK-BR3
*
#

FIGURE 2

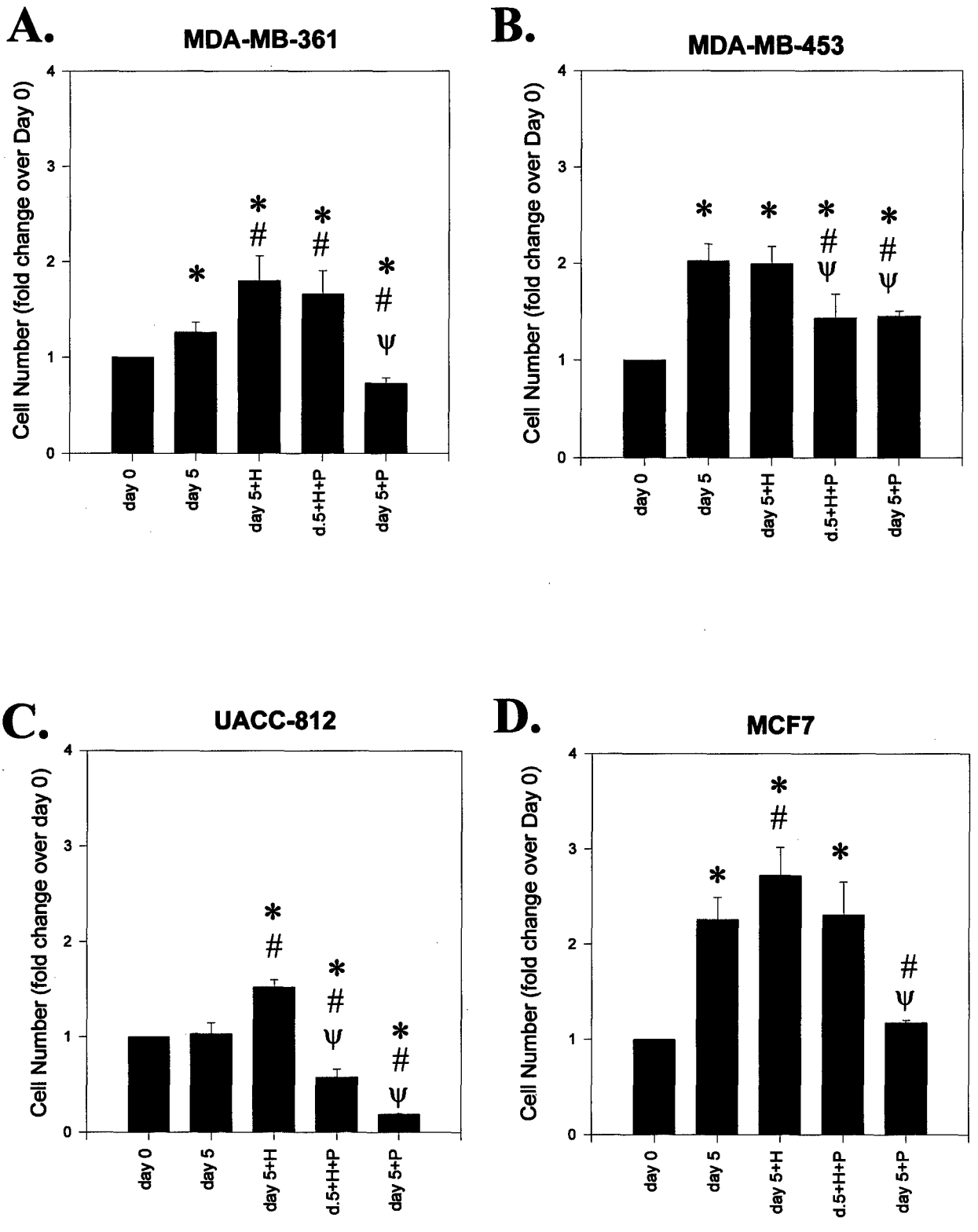
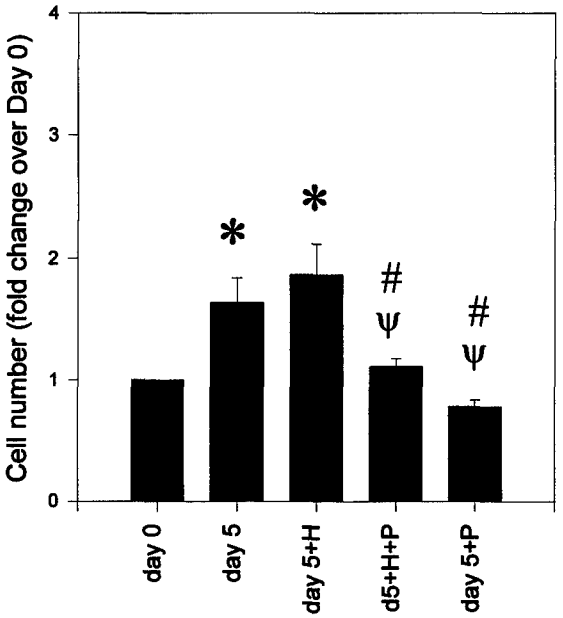

A.
MDA-MB-361
Cell Number (fold change over Day 0)
day 0
day 5
day 5+H
d.5+H+P
day 5+P
B.
MDA-MB-453
Cell Number (fold change over Day 0)
day 0
day 5
day 5+H
d.5+H+P
day 5+P
C.
UACC-812
Cell Number (fold change over day 0)
day 0
day 5
day 5+H
d.5+H+P
day 5+P
D.
MCF7
Cell Number (fold change over Day 0)
day 0
day 5
day 5+H
d.5+H+P
day 5+P

FIGURE 3 (a-d)

E.



F.

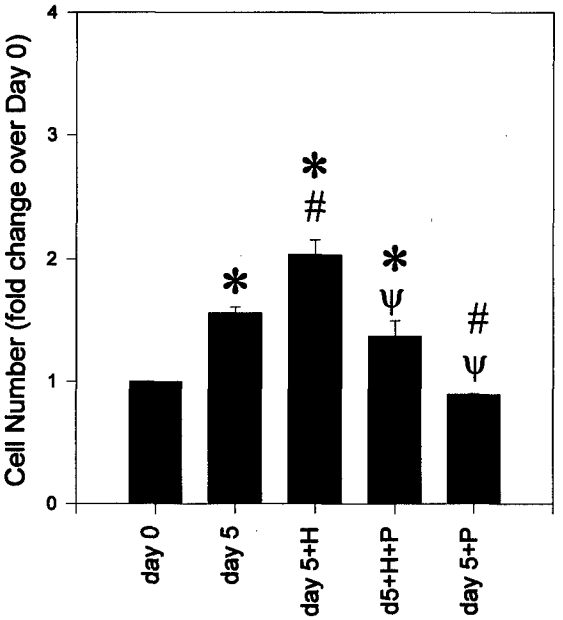


FIGURE 3 (e-f)

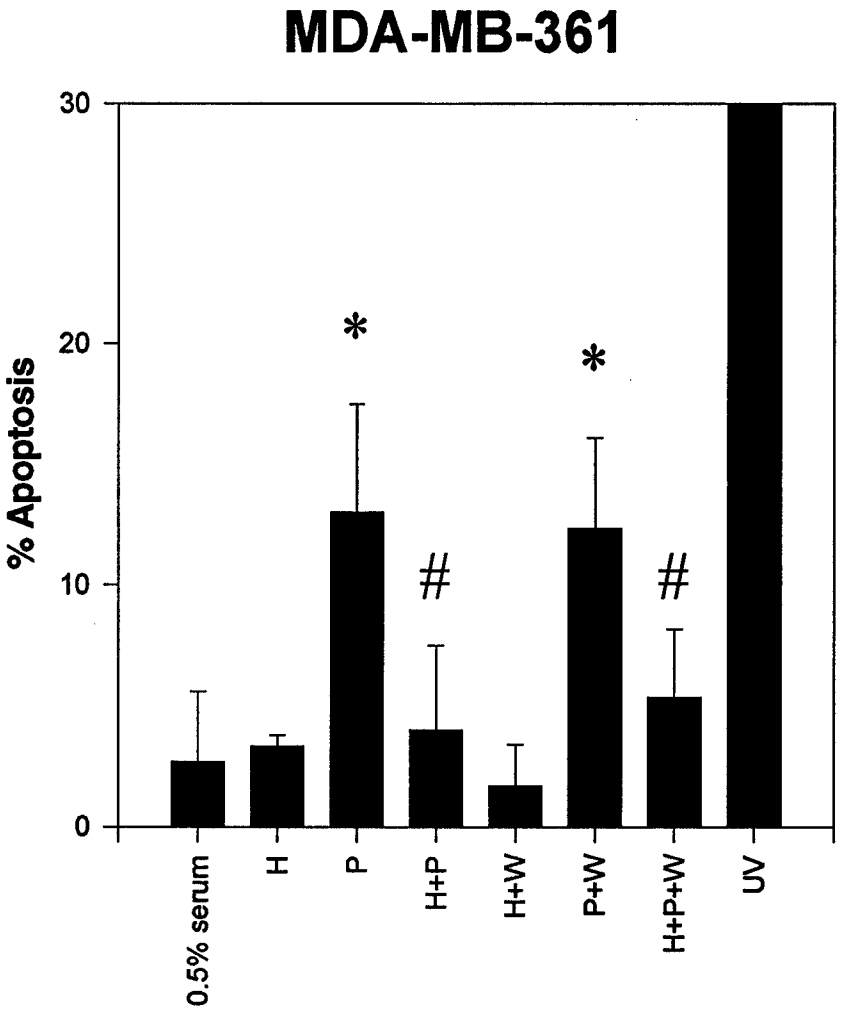
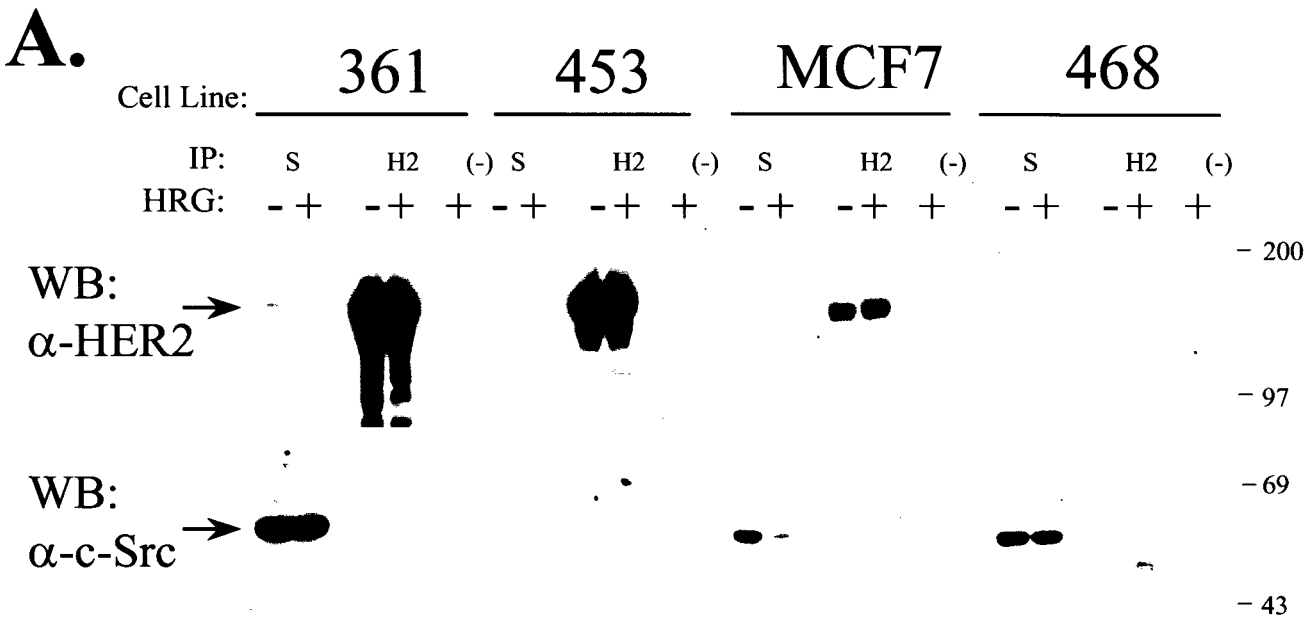


FIGURE 4


A.
Cell Line: 361 453 MCF7 468
IP: S H2 (-) S H2 (-) S H2 (-) S H2 (-)
HRG: - + - + + - + - + + - + - + +
WB: α-HER2
WB: α-c-Src
200
97
69
43

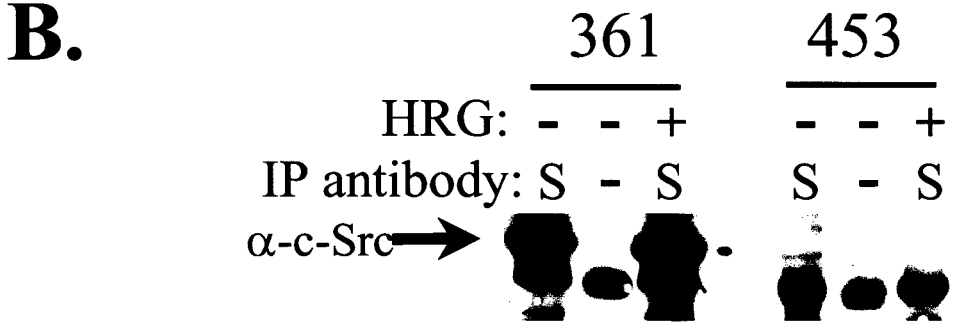

B.
361 453
HRG: - - + - - +
IP antibody: S - S S - S
α-c-Src

FIGURE 5 (a-b)

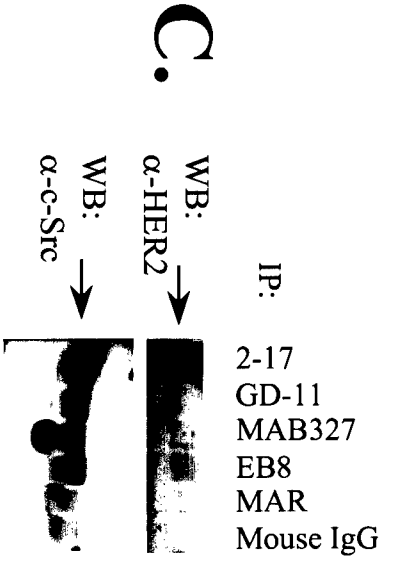


FIGURE 5 (c)

Sample:
Normal Breast
Tumor 156
Tumor 263
Tumor MichN1
IP:
S H1 H2 H3 (-)
S H1 H2 H3 (-)
S H1 H2 (-)
S H1 H2 H3 (-)
kDa
WB: α-HER2
WB: α-c-Src
200
97
69
43

FIGURE 6

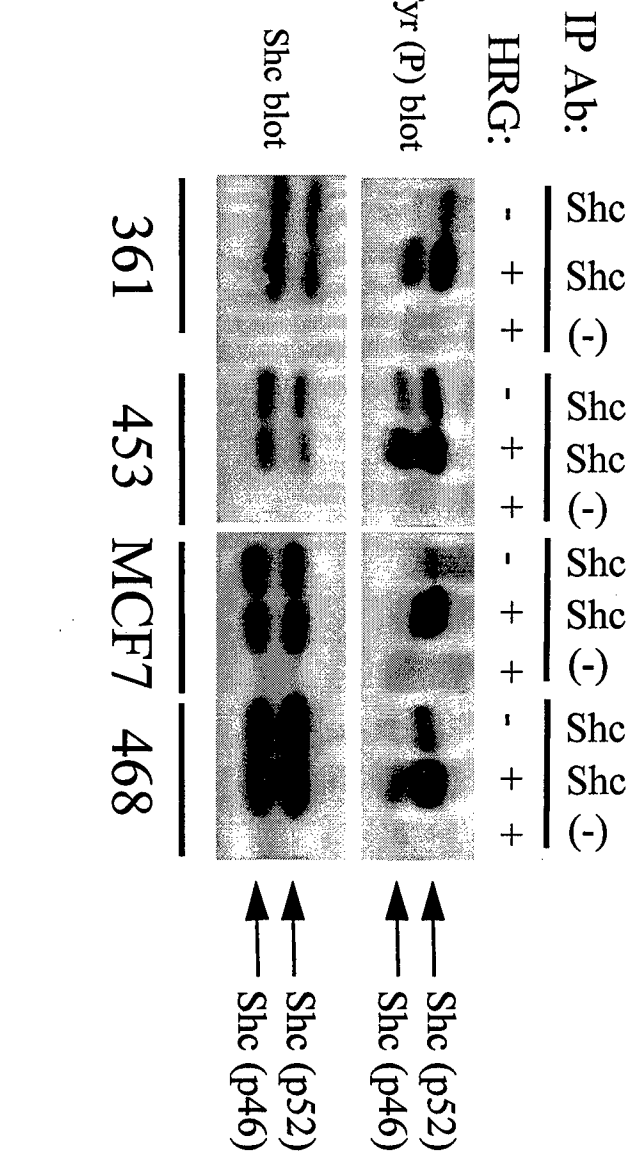

IP Ab:
HRG:
Shc
Shc
(-)
Tyr (P) blot
Shc blot
361
453
MCF7
468
Shc (p52)
Shc (p46)

FIGURE 7

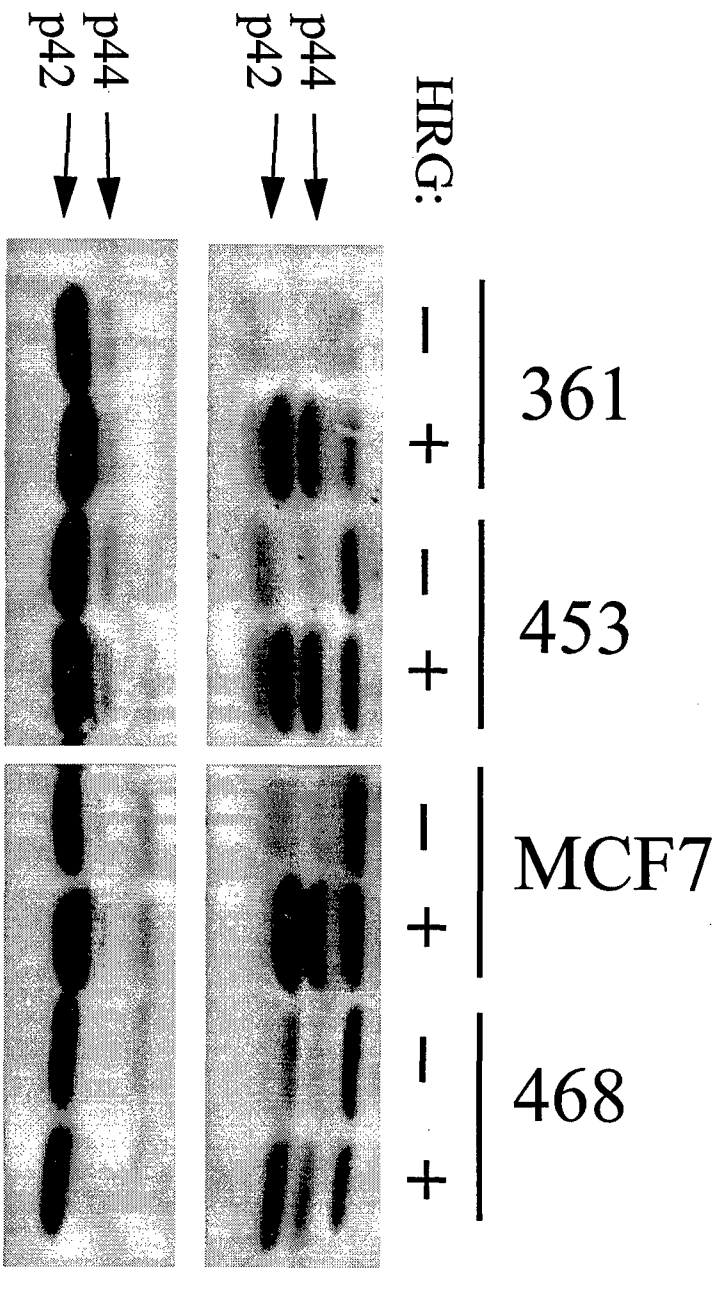


FIGURE 8

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

DAMD17-94-J-4413	ADB261602
DAMD17-96-1-6112	ADB233138
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